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Myofibroblast Differentiation Is Induced in Keratinocyte-Fibroblast Co-Cultures and Is Antagonistically Regulated by Endogenous Transforming Growth Factor-β and Interleukin-1

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In wound healing epidermal-dermal interactions are known to regulate keratinocyte proliferation and differentiation. To find out how fibroblasts respond to epithelial stimuli, we characterized fibroblasts in monolayer co-culture with keratinocytes. On co-culture numerous extracellular matrix- and smooth muscle cell-associated gene transcripts were up-regulated in fibroblasts, suggesting a differentiation into myofibroblasts. Increased α-smooth muscle actin (α-SMA) protein expression in co-cultured fibroblasts started at approximately day 4, was serum-independent, but required endogenous transforming growth factor (TGF)-β. In co-cultures, TGF-β neutralizing monoclonal antibody strongly reduced α-SMA induction. Endogenous TGF-β production and activation were increased at 24 and 48 hours, requiring, like α-SMA induction, close keratinocyte-fibroblast proximity. As myofibroblast differentiation only started after 4 days, we analyzed the presence of endogenous inhibitors at early time points. Blocking keratinocyte-derived interleukin (IL)-1 using IL-1 receptor antagonist, α-SMA expression in co-cultures was potentiated. Conversely, adding exogenous IL-1α completely suppressed endogenous α-SMA induction. In co-cultured fibroblasts strong nuclear factor-κB binding activity was observed from 2 hours, decreasing at 2 and 4 days, suggesting an early, IL-1-mediated inhibition of TGF-β signaling in co-cultured fibroblasts. This biphasic differentiation event is regulated by the balance of endogenous TGF-β and IL-1 activity and is reminiscent of myofibroblast differentiation at early and later stages of wound healing. ***(Am J Pathol 2004, 164:2055–2066)***

Epithelial-mesenchymal interactions play a crucial role in cutaneous tissue repair, tumor invasion, and skin development. In development, ectodermal cells depend on instructive signals from the underlying mesenchyme to first commit to the epithelial lineage and, subsequently, form a fully differentiated epidermis with skin appendages.[1,2] In adult skin, epithelial-mesenchymal interactions are involved in maintaining the epidermal barrier function through tightly regulating the rate of keratinocyte proliferation and subsequent suprabasal keratinization steps. Indeed, cues exchanged through those interactions are critical to skin repair and development, as illustrated by the biology of the keratinocyte growth factor/keratinocyte growth factor receptor system.[3–6]

A paracrine epithelial-mesenchymal stimulatory loop has been proposed that summarizes most findings in cutaneous epithelial-mesenchymal interactions.[7] For fibroblasts, however, a comprehensive picture is still missing, particularly regarding the molecular changes occurring after stimulation by keratinocytes. Fibroblasts are viewed as resting in normal skin, but they can become activated, eg, in tissue repair. On injury, fibroblasts proliferate and migrate into the wounded area, synthesize new connective tissue, and contract it to facilitate wound closure. Fibroblasts undergo extensive changes during wound healing and some fibroblasts start to express smooth muscle cell markers such as α-smooth muscle actin (α-SMA) resulting in a phenotype referred to as myofibroblasts.[8,9] The expression of such smooth muscle-related genes in myofibroblasts correlates with an increased capacity to contract tissue, as demonstrated for α-SMA.[10,11] In early granulation tissue only few myofibroblasts are present, they appear in large numbers at later stages, arranged in layers parallel to the skin surface. Myofibroblasts disappear after completion of repair, possibly through selective apoptosis.[12] Although the biology of myofibroblast differentiation *in vivo* remains to be precisely defined, transforming growth factor (TGF)-βs in

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conjunction with the expression of ED-A fibronectin have been identified as key regulators *in vitro*.[13,14]

In the complex wound environment, fibroblasts are subjected to signals from many different cell types. After the initial inflammatory phase, mostly driven by proinflammatory cytokines such as interleukin (IL)-1 and TNF-α from mononuclear cells, keratinocyte-fibroblast interactions become prominent. In fact, keratinocytes have some potential to complement the role of mononuclear cells with regard to synthesis of proinflammatory mediators and growth factors.[15–18]

To characterize keratinocyte-induced changes in fibroblasts, we compared the gene expression pattern of fibroblasts cultured in the presence or absence of keratinocytes. Because of the complexity of *in vivo* models, we chose the keratinocyte-fibroblast monolayer co-culture to define the mechanisms of epithelial-mesenchymal interactions.[19] In this model, mutual gene induction pathways have been identified, forming the basis of fibroblast-dependent paracrine stimulation of keratinocyte proliferation.[16–18]

Materials and Methods

Cell Isolation and Culture

Human dermal fibroblasts (HDFs) were obtained from adult papillary dermis and isolated as previously described.[16] HDFs from explant cultures were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS), 50 μg/ml ascorbic acid, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HDFs were used between passages 6 and 8. Growth-arrested HDF cells (iHDFs) were obtained by γ-irradiation using a dose of 70 Gy.

Normal human keratinocytes were isolated from adult skin as previously described.[16] Keratinocytes were cultured on an irradiated fibroblast feeder layer in FAD medium (DMEM:Ham's F12, 3:1; Gibco BRL) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS, 5 μg/ml insulin, 1 ng/ml epidermal growth factor, 10^{-10} mol/L cholera toxin, and 24 ng/ml adenine. Keratinocytes were used at passage 4 in these experiments.

The human keratinocyte cell line, HaCaT,[20] was kindly provided by Prof. Dr. N.E. Fusenig (Deutsches Krebsforschungzentrum (DKFZ), Heidelberg, Germany). HaCaT keratinocytes were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS, 50 μg/ml ascorbic acid, 2 mmol/L L-glutamine. HaCaT keratinocytes were used between passages 40 and 60. Cultures were set up in plastic cell culture dishes or on Labtek II slides (Nunc, Wiesbaden, Germany) for immunofluorescence studies. Transwell inserts were obtained from BD (Heidelberg, Germany).

Mink lung epithelial cells transfected with the plasminogen activator inhibitor-1 (PAI-1)/luciferase (PAI/L) reporter construct[21] were kindly provided by Dr. D.B. Rifkin (New York University Medical Center, New York, NY). The cells were cultured in DMEM supplemented with 10% FCS, L-glutamine (2 mmol/L), Hepes (10 mmol/L), and geneticin (250 μg/ml). Cells were used between passage 8 and 35.

Antibodies, Growth Factors, and Inhibitors

The mouse anti-α-SMA monoclonal antibody (clone 1A4[22]), the MOPC 21 isotype control and rabbit polyclonal anti pan-cadherin antibodies were purchased from Sigma (Deisenhofen, Germany). Human platelet-derived and recombinant TGF-β1, IL-1 receptor antagonist, IL-1α, and the neutralizing mouse anti-TGF-β1, -2, -3 (clone 1D11) monoclonal antibody were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). Neutralizing rabbit anti-human IL-1α and -β antibodies were purchased from PreproTech (PreproTech-TEBU, Offenbach, Germany). Hybridoma cells expressing the mouse anti-human integrin α1 monoclonal antibody (clone TS 2/7.1.1[23]) were obtained from American Type Culture Collection (Manassas, VA). The anti ED-A fibronectin (clone IST-9[14]) and collagen IV (clone M3F7) monoclonal antibodies were kindly provided by Prof. Dr. L. Zardi (University of Genua, Genua, Italy) and the Developmental Study Hybridoma Bank (University of Iowa, Iowa City, IA), respectively. Cy3-coupled sheep anti-mouse IgG was obtained from Sigma. Horseradish peroxidase-coupled rabbit anti-mouse IgG was purchased from DAKO Diagnostika (Hamburg, Germany). Alexa 594-nm goat anti-mouse and Alexa 488-nm-coupled goat anti-rabbit IgG were purchased from Molecular Probes (Göttingen, Germany).

Co-Culture Experiments

For establishment of co-cultures, iHDFs or HDFs were plated at a density of 1.5×10^4 cells/cm^2 in 15-cm tissue culture dishes (2.5×10^6 cells/dish) or in six-well plates (1.25×10^5/well), and incubated in DMEM and 10% FCS for 24 hours. Normal human keratinocytes or HaCaT cells were added, at a 1:6 ratio (keratinocytes:fibroblasts), yielding a density of 2.5×10^3 keratinocytes/cm^2, and allowed to attach overnight. For co-cultures with normal human keratinocytes FAD medium containing 10% FCS was used and for co-cultures with HaCaT cells DMEM with 10% FCS or 0.5% FCS as indicated. Serum-free medium consisted of DMEM supplemented with insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenious acid (6.25 ng/ml), bovine serum albumin (BSA) (1.25 mg/ml), linoleic acid (5.35 μg/ml) (ITS+ supplement, BD, Heidelberg, Germany). To physically separate keratinocytes from fibroblasts in co-cultures, HaCaT cells were seeded in the upper compartment of transwell chambers and fibroblasts plated in the lower compartment. For growth factor stimulation or inhibition experiments, after HaCaT keratinocyte attachment, cultures were washed in DMEM without FCS followed by addition of fresh DMEM/0.5% FCS containing growth factors or inhibitors at the indicated concentration. Medium was subsequently replaced every 2 days.

Isolation of Fibroblasts from the Keratinocyte-Fibroblast Co-Cultures

Two methods of fibroblast separation were used. The first method used immunoseparation using anti-α1-integrin mouse monoclonal antibody bound to Dynal 450 sheep anti-mouse IgG paramagnetic beads (Dynal, Hamburg, Germany). Cultures were washed and the paramagnetic beads were added in phosphate-buffered saline (PBS)/1% BSA in a sixfold excess (beads:fibroblasts). After binding and inspection under the phase contrast microscope to verify specificity of labeling, cells were washed and trypsinized. The keratinocyte-fibroblast suspension was separated by magnetic force retaining fibroblasts. Alternatively, the co-cultures were incubated in PBS-ethylenediaminetetraacetic acid (0.05% w/v) with gentle dislodging of the mesenchymal cells, selectively separating fibroblasts from the still attached keratinocytes.[16]

cDNA Microarray Chip Analysis

iHDFs were immunoseparated from primary keratinocytes as described above and the efficiency was verified by replating aliquots of the isolated cell population on a Petri dish. Contaminating keratinocytes accounted for less than 1% of the isolate. Total RNA was isolated according to the acid guanidinium isothiocyanate-acid phenol protocol.[24] Polyadenylated RNA was obtained according to the manufacturer's instructions with Dynabeads Oligo (dT)25 (Dynal). Poly-A^+ RNA samples (7.5 μg each) were then analyzed using the human GEM IV cDNA microarray (Genome Systems-Incyte Pharmaceuticals, Palo Alto, CA).

Northern Blotting

Total RNA was isolated from immunoseparated fibroblasts with Qiagen RNA columns (Qiagen, Hilden, Germany). Ten μg of total RNA were size fractionated and blotted onto nylon membranes. The Readiprime II random-labeling system from Amersham Pharmacia Biotech (Freiburg, Germany) was used to label the following cDNA probes: collagen (I)-α1, a 1400-bp *Eco*RI cDNA fragment; PAI-1 probe (kindly donated by Prof. Dr. K. Preissner, Giessen, Germany); IL-6, a 560-bp *Eco*RI/*Pst*I fragment (kindly provided by Dr. L.T. May, New York, NY); MCP 1 cDNA (gift from Dr. T. Yoshimura, National Cancer Institute, Bethesda, MD); and GAPDH, a 1400-bp *Eco*RI fragment (kindly provided by Dr. E. Schwarz, DKFZ, Heidelberg, Germany). Hybridization was performed using Hybrimax solution (Ambion, Huntingdon, UK). Radioactive signals were visualized on X-ray films.

Electrophoretic Mobility Shift Assays (EMSAs)

Confluent fibroblast cultures were stimulated by seeding 5×10^3 HaCaT cells/cm^2 (2 and 4 day values). For short-term stimulations, HaCaT cells were seeded onto Cytodex 3 microcarrier beads (Sigma) and HaCaT keratinocytes on the microcarrier beads were added to the fibroblasts cultures allowing direct cell-cell contact. Parallel fibroblast cultures without HaCaT cells served as control. After 2, 4, and 6 hours or after 2 and 4 days, fibroblasts were separated from the keratinocytes either by removing the beads or by PBS/ethylenediaminetetraacetic acid treatment. Nuclear extracts were prepared according to Dignam and colleagues.[25] Each nuclear extract containing 5 μg of protein was preincubated with poly (dI-dC) for 15 minutes. To test for nuclear factor (NF)-κB binding activity the mixture was then incubated with a ^{32}P-labeled double-stranded 30-bp probe from the mouse κ light chain enhancer containing one NF-κB site.[26] Samples were run on a 4% polyacrylamide gel and analyzed by autoradiography.

Detection and Quantification of α-SMA Protein

For immunofluorescence studies, cells were cultured on Labtek II slides and fixed in 100% methanol. After blocking (PBS-10% FCS/1 hour at room temperature), anti-α-SMA antibody (1:400/PBS-1% FCS) was incubated for 1 hour at room temperature or overnight at 4°C. To identify keratinocytes, a Rb anti-pan-keratin polyclonal preparation (1:250, DAKO) was co-incubated. After three PBS washes Alexa 488 goat anti-rabbit (1:500) and Alexa 594 goat anti-mouse (1:500) were incubated for 1 hour. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (1 μg/ml). After washes, slides were mounted using FA mounting fluid (BD, Heidelberg, Germany). A Nikon Eclipse 800 microscope coupled to a digital camera (Nikon, Düsseldorf, Germany) in conjunction with image analysis software (Lucia G MV1500; Laboratory Imaging, Czechoslovakia) was used.

For Western blotting, PBS/ethylenediaminetetraacetic acid-separated iHDFs from co-cultures, as well as control iHDFs, were lysed in RIPA buffer and protein concentration determined using the BCA kit (Pierce, Rockford, IL). Samples (2.5 to 5 μg protein per lane) were resolved on duplicate 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One gel was blotted, the other was stained to confirm equal loading. Transfer efficiency was verified by using Ponceau Red staining (Sigma). After blocking [PBS/5% (w/v) nonfat milk powder/0.5% Tween 1 hour at room temperature] 1:3000 diluted mouse anti-α-SMA was added for 1 hour followed by three washes in PBS-0.5% Tween (v/v). Bound primary antibody was detected using peroxidase-coupled Rb anti-mouse (1:1500) and subsequent chemiluminescence (ECL; Amersham/Pharmacia Biotech, Freiburg, Germany). Chemiluminescent signals were visualized on X-ray films.

PAI-1/Luciferase (PAI/L) TGF-β Bioassay

The PAI/L bioassay for active TGF-β was performed as described.[21] For the PAI/L assay, a truncated TGF-β-inducible PAI-1 promoter had been fused to a firefly luciferase reporter gene and subsequently transfected

Table 1. Changes in Gene Expression in iHDF Co-Cultured with Primary Keratinocytes

Proteases and inhibitors			Extracellular matrix components		
Fold	Gene	GenBank Id	Fold	Gene	GenBank Id
Up-regulated genes					
5.9	*PAI 1* (PI)	X04429	5	*Collagen V alpha 3*	AI740960
			4.9	*Collagen IV alpha 1*	M26576
			4.9	*Hyaluronan synthetase 2* (PG)	W49820
			3.4	*Lysine hydroxylase 2*	NM_000935
			2.8	*Transglutaminase 2*	AL031651
			2.6	*Collagen V alpha 1*	M76729
			2.3	*Collagen I alpha 1*	AW577407
			2.3	*Tenascin C* (GP)	X78565
			2.1	*Decorin* (PG)	M14219
			2	*Collagen VI alpha 3*	NM_004369
			2	*Syndecan 2* (PG)	AW951095
Down-regulated genes					
4.03	*Cathepsin K* (P)	NM_000396	17	*Fibulin 4*	U03877
3.04	*Cathepsin L* (P)	AI041851	8.04	*Testican* (PG)	AF231124
3.01	*Cathepsin L2*(P)	AB001928	7	*Tenascin XA* (GP)	U24488
			5	*Fibulin 1*	AW630695
			3.01	*Glypican 1*	NM_002081
			2.02	*Thrombospondin 2* (GP)	NM_003247

Cells were cultured for 4 days in FAD-10% FCS. RNA was isolated from control iHDF and co-cultured iHDF after immunoseparation via their unique α1-integrin expression. Differential mRNA expression was analyzed using a Genome Systems cDNA microarray chip. More than 100 genes of ~7200 tested, were found to be differentially regulated. The genes have been clustered according to their potential function. PI, protease inhibitor; PG, proteoglycan; GP, glycoprotein; P, protease.

into mink lung epithelial cells generating a highly sensitive, TGF-β-responsive cell line. The PAI/L assay measures active TGF-β1, -2, and -3, and the detection limit is ~5 pg/ml.[21]

Mink lung epithelial cells were plated into 96-well plates (1.6×10^4/100 μl/well) in complete DMEM and incubated for 4 hours at 37°C. Then, the serum-containing medium was replaced with 100 μl of the TGF-β-containing, serum-free conditioned media [DMEM containing 0.1% pyrogen-poor BSA (PAA Laboratories, Cölbe, Germany), Hepes (10 mmol/L), and L-glutamine (2 mmol/L); DMEM-BSA medium]. To generate standard curves for TGF-β activity, serial dilutions of recombinant human TGF-β1 (0 to 500 pg/ml) in DMEM-BSA medium were added to the cells in place of conditioned media. Conditioned media and TGF-β standards were incubated in triplicate with the cells overnight at 37°C. The cells were then washed in PBS and lysed in 100 μl of luciferase cell lysis buffer (BD Biosciences, Heidelberg, Germany) for 20 minutes at room temperature. Forty-five μl of the lysates were transferred into a Microlite 96-well plate, and luciferase activity in the lysates was determined using a MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA). Luciferin was obtained from Biosynth (Staad, Switzerland).

Results

HDFs Co-Cultured with Human Keratinocytes Alter Their mRNA Expression Profile

To characterize the response of fibroblasts to interaction with epidermal cells, we compared the mRNA expression pattern of irradiated human dermal fibroblasts (iHDFs) cultured in the presence or absence of normal human keratinocytes. After co-culture for 4 days, iHDFs were isolated from keratinocytes via their exclusive α1-integrin expression by anti-α1-integrin-specific paramagnetic bead separation. This yielded a very pure iHDF population, as judged from replated aliquots, with less than 1% keratinocyte contamination. After poly-A^+ RNA extraction from co-cultured as well as control iHDFs, we compared the mRNA expression pattern using a Genome Systems cDNA microarray chip.

Among the 7200 genes examined, more than 100 were either significantly up- or down-regulated in the co-cultured iHDFs, compared with iHDFs from mono-cultures. Induction or down-regulation by a factor of two was considered significant (Table 1).[27] The majority of the regulated genes could be grouped into four categories: growth factors and inflammatory mediators, extracellular

Table 1. *Continued*

Cell-cell signaling associated			Cytoskeleton associated		
Fold	Gene	GenBank Id	Fold	Gene	GenBank Id
11	*ENA 78*	NM_002994	3.3	*SM22 alpha*	AF013711
9.7	*MCP1*	M26683	3	*Integrin alpha 1*	X68742
7.4	*Activin A*	M13436	2.9	*Tropomyosin 2 beta*	M75165
5.3	*LTBP 1*	NM_000627	2.7	*Tropomyosin 1 alpha*	Z24727
4.2	*Interleukin 6*	M54894	2.5	*VASP*	NM_003370
3.7	*CTGF*	U14750	2.5	*Calponin 1 (Basic)*	NM_001299
3.3	*HB-EGF*	AC004634	2.4	*Smooth muscle actin alpha2*	X13839
2.9	*Endothelin 1*	NM_001955	2.3	*n-chimaerin*	X51408
2.9	*Angiopoetin 1*	D13628	2.1	*MLCK*	AW951177
2.6	*WNT 5A*	L20861	2.1	*Integrin alpha 5*	NM_002205
2.6	*LIF*	X13967			
2.4	*COX 2*	D28235			
2.3	*GCSF*	M17706			
2.1	*WNT 2*	NM_003391			
2	*Dickkopf 3*	AB033421			
6.08	*TGF beta receptor III*	AJ251961	2.09	*Integrin alpha 4*	NM_000885
5.06	*Prostaglandin F Receptor*	NM_000959			
3.05	*Follistatin*	NM_006350			
2.08	*GP130 Receptor*	NM_002184			
2.07	*Osteoprotegerin Receptor*	U94332			
2.03	*Prostacyclin*	D83402			
2.02	*Toll Receptor 4*	NM_003266			
2.02	*EGF Receptor*	NM_003247			
2.01	*HGF Receptor*	X54559			
2.01	*MCSF*	M11296			
2	*Stromal cell-derived factor*	L36033			

matrix (ECM)-related constituents and enzymes, proteases and their inhibitors, and cytoskeletal components.

Many genes associated with inflammatory responses, eg, the chemokines *ENA-78*, and *MCP-1*, *IL-6*, *LIF*, *G-CSF*, *M-CSF*, or *COX2* were up-regulated in co-cultured iHDFs. Increased expression of genes encoding profibrotic and fibroblast-activating growth factors was observed. Thus, expression of activin A, a TGF-β family member, *CTGF*, a downstream mediator of TGF-β, and *endothelin 1* were all up-regulated. In the ECM and proteases category, several interstitial collagen genes, *collagen (I) $\alpha 1$*, *collagen (V) $\alpha 1$* and $\alpha 3$, and *collagen (VI) $\alpha 3$* were induced as well as the basement membrane *collagen (IV) $\alpha 1$*. Several other constituents of the ECM, *tenascin C*, *decorin*, and *syndecan 2* were induced, whereas *fibulin 1* and *4*, *glypican*, and *thrombospondin 2* were down-regulated. mRNA levels of collagen-modifying enzymes, *lysyl hydroxylase* and *transglutaminase 2*, which modify ECM molecules and stabilize the ECM by introducing cross-links, were increased. *Plasminogen activator inhibitor-1* (PAI-1), a well-known TGF-β target gene, was strongly up-regulated, whereas several potent proteases, *cathepsins L*, *L2*, and *K* were down-regulated. In iHDFs co-cultured with keratinocytes, genes associated with the contractile apparatus of smooth muscle cells were also induced, eg, *α-SMA*, *myosin light chain kinase, calponin*, as well as *tropomyosin 1* and *2*, as were genes involved in the formation of stress fibers and focal adhesions, eg, *vasodilator-stimulated phosphoprotein*, *integrin $\alpha 1$* and $\alpha 5$ chains, and *SM22-α*.

Similar results were obtained with HaCaT cells, a human keratinocyte cell line, using Northern blotting. In fibroblasts co-cultured with HaCaT cells, profibrotic markers [*PAI-1* and *collagen (I) $\alpha 1$*] were found to be up-regulated (Figure 1). The induction of IL-6 is consis-

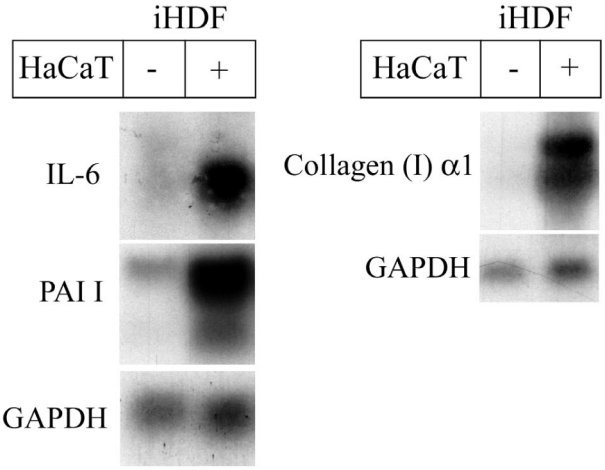

Figure 1. Differential gene expression in fibroblasts on co-culture with HaCaT keratinocytes. RNA from iHDFs cultured in the absence (−) or presence of HaCaT cells (+) was prepared after 4 days. Ten μg of total RNA was hybridized to probes indicated on the left. The experiments were performed with three different fibroblast strains each revealing a clearly visible up-regulation of IL-6, PAI-1, and collagen type I in the co-cultured iHDF strains. For collagen type I, the exposure time was extremely short, hence the very weak band in iHDF without HaCaT cells. These results correlated well with the cDNA microarray hybridization experiment.

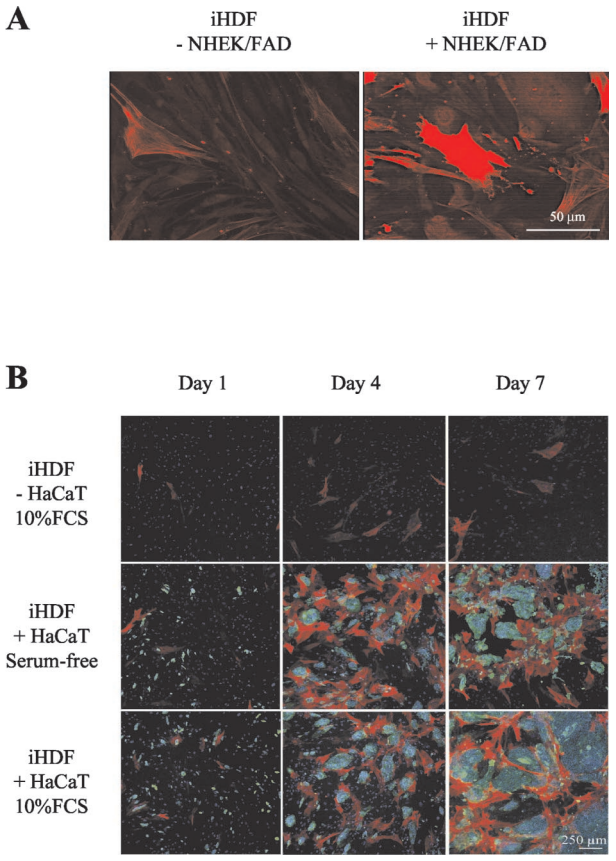

Figure 2. A: α-SMA protein induction in co-cultured iHDFs. iHDFs were cultured in the absence or presence of primary normal human epidermal keratinocytes (NHEKs) for 6 days and stained for α-SMA expression. **B:** Time course experiments of α-SMA expression were done in iHDFs cultured with or without HaCaT keratinocytes in medium containing either 10% or serum-free medium, indicated on the left. Cultures were immunostained for α-SMA expression (red) and pan-keratin (green) after 1, 4, and 7 days. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue). At approximately day 4, a clear increase of α-SMA-positive iHDF cells was seen in co-cultures. After 7 days, the majority of iHDFs expressed α-SMA. Myofibroblast differentiation in the co-cultures appeared to be independent of exogenous growth factors.

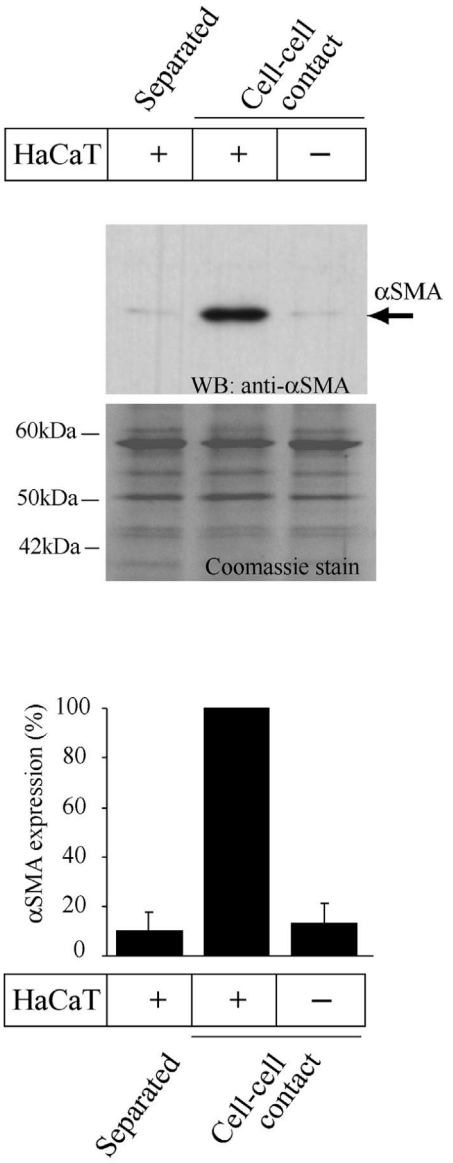

Figure 3. Top: α-SMA induction in co-cultured iHDFs requires close proximity of keratinocytes and fibroblasts. iHDFs were co-cultured with HaCaT cells in transwell chambers preventing direct cell-cell contact, yet, permitting interactions via diffusable mediators (**left lane**). Monolayer co-cultures with direct cell-cell contact in the presence (**middle lane**) or absence of HaCaT cells (**right lane**) in medium containing 10% FCS. After 7 days, iHDFs were isolated from the co-cultures and α-SMA expression was detected by Western blotting. A Coomassie-stained parallel gel is shown at the **bottom**. The experiment shown represents a typical blot obtained in three independent experiments. Data represent the mean value ± SEM of three independent experiments. α-SMA levels in co-cultures were set at 100%.

tent with previous experiments involving keratinocyte-fibroblast co-cultures.[16]

Co-Culture with Keratinocytes Induces Myofibroblast Differentiation

The mRNA expression analysis showed an up-regulation of smooth muscle cell-associated markers and ECM-related genes in co-cultured fibroblasts, indicating that iHDFs responded to the co-culture conditions with a differentiation into myofibroblasts. Because α-SMA is considered to be characteristic for myofibroblast differentiation,[9] we used the well-characterized 1A4 monoclonal antibody to detect α-SMA protein.[22] We first investigated co-cultures with primary keratinocytes and fibroblasts for emergence of α-SMA-positive myofibroblasts. Indeed, there was an increase of α-SMA expression in co-cultured mesenchymal cells at 6 days but not in fibroblast control cultures (Figure 2A). To investigate myofibroblast differentiation in more detail and under more standardized conditions, we used HaCaT keratinocytes because this cell line is very well characterized *in vitro* as well as in tissue transplants.[20,28,29] After 7 days of culture, using immunofluorescence and Western blotting to detect α-SMA protein, a more than sixfold increase in α-SMA expression was seen in iHDFs co-cultured with HaCaT keratinocytes as compared to iHDFs cultured alone (Figure 2B, top *versus* middle and lower row; Figure 3, middle lane), confirming the results of our microarray analysis. Myofibroblast differentiation was also observed in proliferation competent fibroblasts. The ratio of HaCaT kera-

tinocytes/fibroblasts had to be adjusted to 1:3, respectively, to avoid overgrowth of the HaCaT cells. The extent of α-SMA-expressing cells as well as the kinetics of appearance were comparable to growth-inhibited, irradiated fibroblasts (data not shown). Furthermore, staining for ED-A fibronectin as well as for collagen IV revealed increased deposition of ECM components in co-cultures compared with fibroblast monocultures. For ED-A fibronectin a diffuse deposition pattern was observed within the fibroblast populated areas but not the keratinocyte colonies, whereas collagen IV staining was confined to the zone surrounding the keratinocyte colonies with close proximity to fibroblasts (data not shown).

Fibroblast to Myofibroblast Differentiation Is Independent of Exogenously Added Factors but Requires Close Proximity to Keratinocytes

The myofibroblast differentiation in response to co-culture with keratinocytes was also observed in the absence of serum pointing to an endogenous regulatory mechanism. Throughout the 7 days of culture, the fraction of α-SMA-expressing iHDFs did not differ in co-cultures performed in the presence of either 10% serum or serum-free DMEM supplemented with insulin, transferrin, selenium, BSA, and linoleic acid (Figure 2, middle and lower row). However, α-SMA-expressing myofibroblasts were scarce before day 4 in co-cultures.

In addition, we noted a strong dependence on cell proximity of both cell populations. Stainings with anti pan-cadherin antibodies suggested the presence of some junctional complexes between HaCaT keratinocytes and surrounding fibroblasts after 7 days of co-culture. Furthermore, when keratinocytes were seeded in cloning rings onto iHDFs, only fibroblasts adjacent to the keratinocyte colonies started to express α-SMA (data not shown). When cultured physically separated using transwell culture inserts, the number of α-SMA-positive fibroblasts did not increase and was comparable to control cultures (Figure 3, left lane).

Myofibroblast Differentiation in Keratinocyte-Fibroblast Co-Cultures Requires Endogenous TGF-β Activity

TGF-β is a well known inducer of myofibroblast differentiation.[13] In addition, the profibrotic gene expression pattern and the induction of PAI-1 in these co-cultures suggested the generation of active TGF-β. We therefore added a monoclonal antibody neutralizing all three isoforms of TGF-β to the co-cultures and used α-SMA as a surrogate marker for fibroblast-myofibroblast differentiation. More than 60% of the α-SMA induction could be blocked by the neutralizing TGF-β antibody (Figure 4, left lane). The MOPC 21 isotype control had no inhibitory effects on α-SMA expression levels (data not shown). We determined TGF-β levels in keratinocyte-fibroblast co-cultures. Using the PAI-1/luciferase (PAI/L) bioassay, we measured latent and activated TGF-β in supernatants of

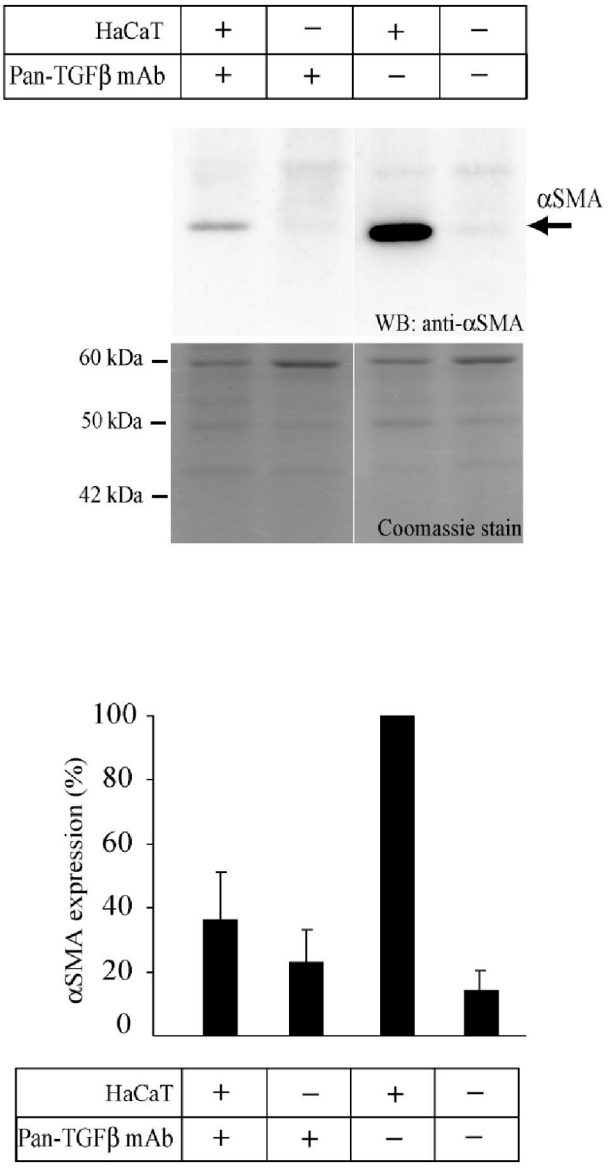

Figure 4. Top: Inhibition of endogenous TGF-β represses α-SMA induction in co-cultured iHDFs. iHDFs were cultured in the presence (+) or absence (−) of HaCaT cells for 7 days. A neutralizing monoclonal antibody against the TGF-β isoforms 1, 2, and 3 (10 μg/ml, **left two lanes**) or plain culture medium (0.5% FCS, **right two lanes**) were added to the co-cultures. Medium was changed every 2 days. α-SMA expression was assessed by Western blotting and the MOPC 21-isotype control antibody had no effect in HaCaT-iHDFs or iHDF control cultures (data not shown). A Coomassie-stained parallel gel is shown at the **bottom**. A representative blot is shown out of three independent experiments. Data represent the average ± SEM of three independent experiments. α-SMA levels in co-cultures were set at 100%. The neutralizing TGF-β antibody inhibited α-SMA induction significantly but not completely in co-cultures.

co-cultures with different ratios of HaCaT keratinocytes and fibroblasts as well as in the respective monocultures (Figure 5, left).

Monocultures of HaCaT cells and fibroblasts produced latent TGF-β in the range of 200 pg/ml and 600 pg/ml, respectively (Figure 5, middle). The luciferase signal elicited by the conditioned media in the PAI/L bioassay was almost completely quenched by neutralizing, pan-specific anti-TGF-β antibodies (data not shown) indicating the specificity of the above measurements. Both cell

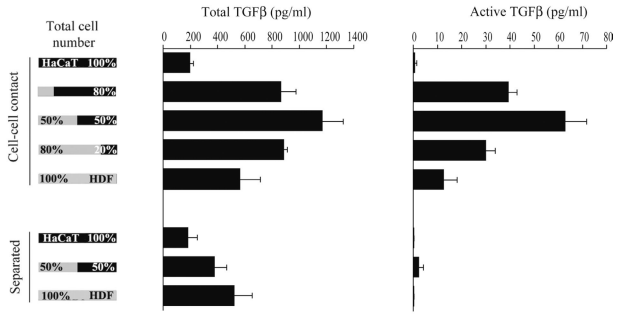

Figure 5. Induction of latent TGF-β production and TGF-β activation in HaCaT-HDF co-cultures. A total of 10^5 cells were plated, at various ratios of HDF to HaCaT cells, into 24-well plates. The relative cell percentages are indicated at the **left**. Cells had direct cell-to-cell contact (**top**) or were separated by transwell inserts (**bottom**). Conditioned media (serum-free DMEM containing 0.1% pyrogen-poor BSA) were collected after 48 hours and analyzed for total (after heat activation, **middle column**) and active (**right column**) TGF-β activity using the PAI/L bioassay. Data represent the mean value ± SEM of two to six experiments each analyzed in triplicate. Efficient latent TGF-β activation only occurred in co-cultures and required direct cell-to-cell contact.

types generated low amounts (< 5 to 10 pg/ml) of activated TGF-β family members (Figure 5, right). In contrast, co-cultures of both cell types produced 32.5 pg/ml of active TGF-β after 24 hours and up to 63 pg/ml after 48 hours with a cell ratio of 50:50 being optimal (Figure 5, right). This indicates that both cell types are required for efficient TGF-β activation. Total TGF-β production in co-cultures was also up-regulated. When HDFs and HaCaT cells were physically separated in the co-cultures using transwell inserts, latent TGF-β activation as well as the induction of total TGF-β production were almost completely abolished (Figure 5, bottom). These results indicate the requirement of close cell proximity for latent TGF-β activation and are consistent with α-SMA induction by endogenous TGF-β.

IL-1 Inhibits Myofibroblast Differentiation in Fibroblasts Co-Cultured with Keratinocytes

Whereas significant TGF-β activity was detected after 24 and 48 hours in co-cultures, α-SMA induction was only clearly detectable on day 4 suggesting an endogenous inhibitor in early stages of co-culture. In keratinocyte-fibroblast co-cultures, keratinocyte-derived IL-1 has been shown to play a crucial regulatory role in the stimulation of gene expression in fibroblasts.[18] Searching for an endogenous inhibitor at early time points of co-culture, we perturbed the action of keratinocyte-derived IL-1 with an excess of IL-1 receptor antagonist (IL-1RA). There was an increase of 60% after 4 days and 50% after 7 days for α-SMA expression in co-cultured fibroblasts compared with co-cultured fibroblasts without IL-1 inhibition (Figure 6A, 4 and 7 day experiments). These data suggest that endogenous IL-1 antagonizes the TGF-β-mediated α-SMA induction in co-cultures. Because activated NF-κB, an IL-1-inducible transcription factor, was shown to inhibit TGF-β signaling[30,31] we determined NF-κB binding activity in co-cultured and control fibroblasts. There was a strong activation of NF-κB in fibroblasts as early as

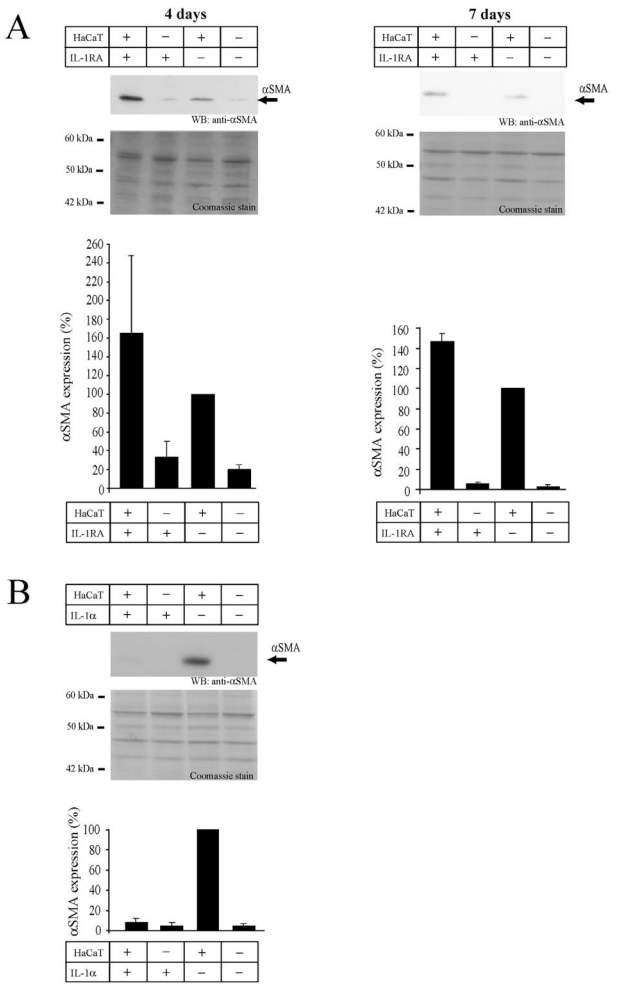

Figure 6. Inhibitory effects of IL-1 on α-SMA expression in HaCaT-iHDF co-cultures. iHDFs were cultured in the presence or absence of HaCaT cells in medium containing 0.5% FCS. IL-1RA (200 ng/ml) (**A**) and IL-1α (0.2 ng/ml) (**B**) were added to cultures. Medium was changed every 2 days. α-SMA expression was assessed by Western blotting. **A:** α-SMA expression was already increased after 4 days of co-culture and remained elevated up to 7 days when IL-1RA was present. The weak band intensity in the 7-day panel (**A**) is because of under-exposure to allow for visualization of up-regulated α-SMA expression in the IL-1RA-treated cultures. A Coomassie-stained parallel gel is shown in each **bottom** panel. Three independent experiments were performed and values represent the average ± SD of three independent experiments. α-SMA levels in co-cultures were set at 100%. Blocking endogenous, keratinocyte-derived IL-1 augmented α-SMA expression, whereas exogenous IL-1α in co-cultures strongly inhibited α-SMA expression.

2 hours after contact with HaCaT cells, which peaked at 6 hours, followed by a significant decline throughout the following 7 days (Figure 7A). Additional experiments showed that NF-κB activation was predominant because of keratinocyte-derived IL-1α. Adding IL-1RA, and neutralizing anti IL-1α antibodies prevented NF-κB activation in the fibroblasts co-cultured for 6 hours. Blocking IL-1β also inhibited NF-κB activation but less efficiently compared with neutralizing anti IL-1α antibodies (Figure 7B). Addition of exogenous IL-1α to co-cultures resulted in an almost complete inhibition of α-SMA induction (Figure 6B, left lane and third lane from left), in line with the observation made with IL-1RA.

To test whether keratinocyte-derived IL-1α inhibited TGF-β-mediated α-SMA induction we stimulated fibro-

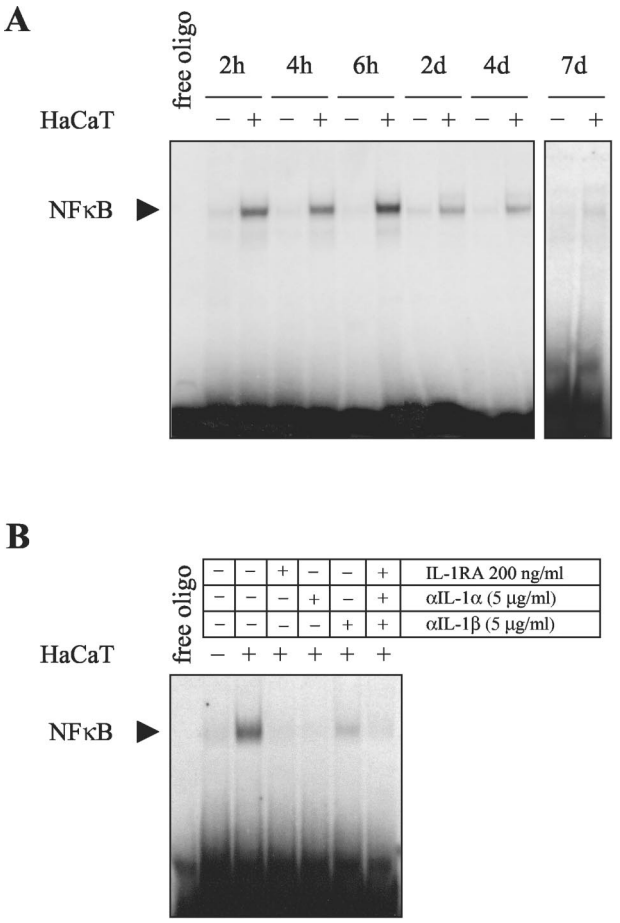

Figure 7. Keratinocyte-derived IL-1 induces NF-κB activation in co-cultured fibroblasts. In **A**, fibroblasts were cultured in the presence or absence of HaCaT cells for different times in medium containing 10% FCS. Nuclear extracts from the mesenchymal cells were analyzed for NF-κB-binding activity by EMSA. The shifted complexes are indicated at the **left**. The 7-day autoradiograph was derived for a separate experiment carrying the 2-hour values as internal standards for exposure times. The EMSA experiments show a strong NF-κB activation in co-cultured fibroblasts, yet, with culture time the signal intensity decreased despite increasing HaCaT cell numbers. In **B**, HaCaT keratinocytes were co-cultured with fibroblasts for 6 hours with or without IL-1RA and neutralizing antibodies as indicated. Fibroblasts alone were included as a negative control. From the neutralization experiments IL-1α seems to be the main inducer of NF-κB activation in co-cultured fibroblasts. The experiments were performed two times independently showing comparable results.

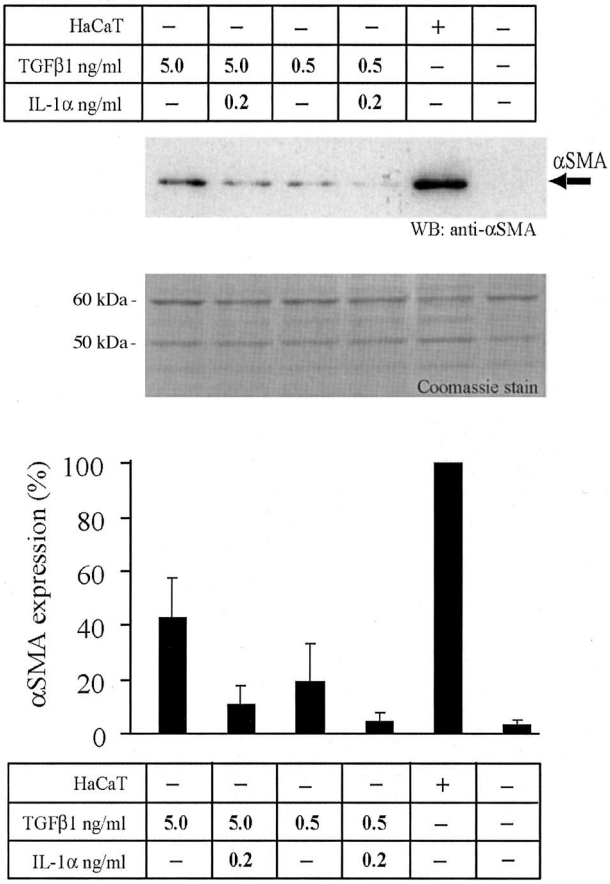

Figure 8. Keratinocyte-derived IL-1 blocks TGF-β-mediated induction of α-SMA in fibroblast monocultures. IL-1α was added to fibroblast monocultures (0.2 ng/ml) stimulated with different concentrations of TGF-β1. Medium was changed every 2 days. α-SMA expression was assessed by Western blotting. Co-stimulation of IL-1 and TGF-β blocks TGF-β-mediated α-SMA induction, suggesting interference with TGF-β-mediated transcription. Values represent the average ± SD of three independent experiments. α-SMA levels in co-cultures were set at 100%.

blast monocultures with TGF-β at 0.5 ng/ml and 5.0 ng/ml with or without a 0.2 ng/ml-IL-1α co-stimulation. In these experiments α-SMA induction by TGF-β was inhibited by ~75% by IL-1α. This was observed for both TGF-β doses used (Figure 8B). Our data indicate that fibroblast differentiation to myofibroblasts is induced by co-culture with keratinocytes and regulated by the balance of endogenous TGF-β and IL-1.

Discussion

Skin development involves epithelial-mesenchymal interactions from early developmental stages onwards. Later in adult life, these interactions still take place and participate in the regulation of skin homeostasis. Metabolic activity of skin cells as well as epithelial-mesenchymal interactions become activated after injury or in skin diseases. Although epithelial-mesenchymal interactions are known to regulate keratinocyte functions, it is not known how they affect fibroblasts.

We chose an *in vitro* model, the keratinocyte-fibroblast monolayer co-culture for this study.[19] This model facilitates the investigation of keratinocyte influences on the co-cultured fibroblast phenotype while omitting superimposed, additional influences present in intact skin or the whole organism. In this model, keratinocytes are seeded on irradiated fibroblasts. Irradiation blocks fibroblast proliferation, thus preventing them from overgrowing keratinocytes, while leaving their metabolic capacity mostly unchanged.[32,33] In this model, mutual gene induction in keratinocytes and fibroblasts has been described initially. Keratinocytes induce fibroblasts to produce growth factors which in turn stimulate keratinocyte proliferation and keratinization.[16,19,34] For subsequent experiments we used the human HaCaT keratinocyte line.[20] This cell line is considered to closely represent human primary keratinocytes.[16,18,20,28,29]

Using this complex tissue culture model, we were able to demonstrate that fibroblasts profoundly modify their

phenotype on co-culture with keratinocytes. First, fibroblasts in co-culture were induced to establish new connective tissue. This was characterized by increased gene expression of ECM constituents such as several collagens, interstitial as well as basement membrane types, numerous proteoglycans and glycoproteins and, finally, enzymes associated with ECM processing and maturation These results are consistent with our previous work demonstrating induction of basement membrane component expression on activation of epithelial-mesenchymal interactions.[35] In addition, increased expression of the protease inhibitor, plasminogen activator inhibitor 1 (PAI-1) together with the decreased expression of several potent proteases points toward decreased proteolytic activity in co-cultures. However, MMP-1 mRNA levels and pro-MMP forms were shown to be induced in keratinocyte-fibroblast co-cultures (unpublished observations).[16] Still, the net effect seems to be an increase in ECM production and deposition as shown here for ED-A fibronectin and collagen IV. Although these experiments do not strictly reflect ECM synthesis rates, only deposited ECM is considered to provide instructive signals to the cells or augment TGF-β activity. Most interestingly, several genes associated with the contractile apparatus of smooth muscle cells such as α-SMA, myosin light chain kinase, and regulators of contraction such as calponin, tropomyosin 1, and tropomyosin 2 were induced in co-cultured fibroblasts. Those two major phenotypic changes suggested that iHDFs co-cultured with keratinocytes differentiated into myofibroblasts.[9,36]

To further define the underlying mechanisms, we monitored α-SMA protein, the marker for myofibroblast differentiation.[9,36] Increased α-SMA protein levels in immunofluorescence and Western blotting confirmed our hypothesis of extensive myofibroblast differentiation in co-cultures, which occurred even in the absence of serum. α-SMA induction only started after 4 days suggesting the involvement of multiple endogenous, possibly paracrine signaling events. It also required close contact between keratinocytes and fibroblasts pointing to mechanisms involving short-range cellular interactions. From stainings for cadherins we obtained evidence for junctional complexes between the two cell types, yet, the relevance for α-SMA and TGF-β induction has to remain open at present. However, whether co-cultured fibroblasts were proliferation competent or growth restricted by irradiation seemed to have little effect on α-SMA induction in this system.

TGF-β family members, TGF-β1, -2, and -3, have been identified as principal inducers of myofibroblast differentiation,[13] making them likely candidates to be up-regulated in our system. Furthermore, gene expression of PAI-1 and CTGF, two genes strongly associated with TGF-β responses, were significantly induced in the co-cultured fibroblasts.[37,38] Indeed, in keratinocyte-fibroblast co-cultures, production and activation of TGF-β were strongly increased, as early as 24 to 48 hours, further pointing to the key role in myofibroblast differentiation in our culture system. Blocking TGF-β activity resulted in a large inhibition of the α-SMA induction observed in fibroblasts on co-culture with keratinocytes. This TGF-β activation in co-cultures is of particular relevance, because cells secrete TGF-βs in a latent form, and extracellular activation of this precursor is believed to represent a major regulatory site of TGF-β activity. Apparently, monocultures do not express the complete machinery required for efficient activation of latent TGF-βs.[39] Close cell-cell contact was also required for activation of latent TGF-β, previously also observed in co-cultures of other cell types.[40–42] This paralleled very well with the α-SMA expression pattern providing a possible explanation for the lack of α-SMA induction observed in transwell cultures. The up-regulation of total TGF-β production in the co-cultures could in part be because of an autoinduction of TGF-β1 gene expression, a mechanism reported in many cell types.[43] We also observed an induction of TGF-β2 mRNA in co-cultured fibroblasts but the mechanism remains elusive.[44]

Still, myofibroblast differentiation could not be completely inhibited through TGF-β neutralization, implying that other growth factors or alternatively the ECM or both could contribute in an auxiliary function. There is evidence that the myofibroblast phenotype is at least in part governed by cell-matrix interactions. For example, ED-A fibronectin deposition, which follows TGF-β stimulation, is required for TGF-β-induced myofibroblast differentiation.[14] In fact, we observed an increase in the deposition of ED-A fibronectin on the fibroblast surface in co-cultures as compared to monocultures (data not shown). Finally, mechanical tension was shown to play a role in inducing myofibroblast differentiation as had been shown for fibroblasts embedded in collagen gels or on plastic dishes.[45,46] However, in all systems TGF-β played a dominant role located up-stream of ECM effects and mechanical forces.

Although active TGF-β was present in the co-cultures at early time points, myofibroblast differentiation occurred late. This prompted us to search for endogenous inhibitors of TGF-β-mediated myofibroblast differentiation present in early stages of co-culture. It is well documented that keratinocytes are the primary source of IL-1 activity in keratinocyte-fibroblast co-cultures.[18] Keratinocyte-derived IL-1 induces the expression of KGF and other growth factors in co-cultured fibroblasts, which in turn stimulate keratinocyte proliferation.[17,18,34] IL-1 levels have been determined in keratinocyte-fibroblast co-cultures in detail.[18] During the first 48 hours high levels of IL-1α (up to 350 pg/10^6 cells/48 hours) and IL-1β (1.450 pg/10^6 cells/48 hours) have been reported. From day 2 onward IL-1 amounts declined (IL-1α, 50 to 70 pg/10^6 cells/48 hours; IL-1β, 170 pg/10^6 cells/48 hours).[18] Furthermore, it has been shown that keratinocytes constitutively express high levels of IL-1 receptor antagonist.[47]

Indeed, we could show in blocking experiments that co-cultured fibroblasts responded to endogenous, keratinocyte-derived IL-1 with NF-κB activation. However, with increasing time of co-culture the response diminished as judged from NF-κB activation levels, which is consistent with measurements of IL-1 levels in co-cultures.[18] Activation of NF-κB has been shown to inhibit TGF-β signaling.[30,31] One study using RelA-deficient and wild-type fibroblasts showed an up-regulation of Smad7

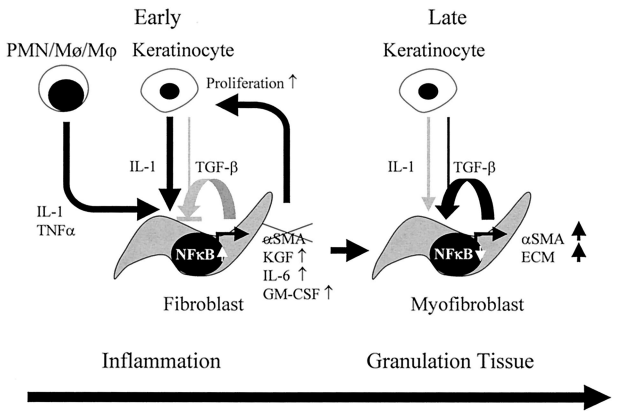

Figure 9. Proposed model for the fibroblast phenotype in the wound environment. A balance of proinflammatory and TGF-β stimuli governs the phenotype of fibroblasts in tissue repair. Fibroblasts initially respond to PMNs, monocytes/macrophages, and keratinocyte-derived IL-1 with activation of NF-κB and subsequently with an up-regulation of IL-1-responsive genes such as KGF, IL-6, and GM-CSF. Although TGF-β activity is present from platelets and activated keratinocyte-fibroblast interactions, NF-κB activation seems to interfere with TGF-β signaling. Later, when the wound environment becomes less proinflammatory dominated, the fibroblasts will respond to TGF-β and differentiate into myofibroblasts as seen by their induction of α-SMA, ECM molecules, and matrix-modifying enzymes. Dominant pathways are shown by **black arrows**. Pathways that are blocked or do not prevail are indicated by **gray arrows**.

after NF-κB stimulation and thus blocking of TGF-β effects.[30] The second study showed in transfection experiments of 293 and HepG2 cells that p300 was limiting for TGF-β effects on several promoter/reporter constructs when p65 was co-transfected or endogenous NF-κB activated.[31] Both studies analyzed Smad7 protein or the Smad7 promoter; the somehow conflicting results might among other things indicate cell type-specific responses associated with the epithelial or mesenchymal cell lineage. Taken together, however, both studies observed NF-κB-dependent inhibition of TGF-β signaling. This could explain why TGF-β-stimulated fibroblast monocultures largely failed to up-regulate α-SMA expression, when co-stimulated with IL-1α and TGF-β. This strongly suggests that, although endogenous TGF-β activity is crucial for myofibroblast differentiation in the co-culture system, its action is blocked by proinflammatory cytokines in early culture stages. Intriguingly *in vivo*, myofibroblasts appear late in the course of wound healing, ie, little α-SMA expression is observed before 3 days after wounding, with a strong up-regulation of expression being observed at 6 days.[10] This contrasts with the early release of active TGF-β from platelets in the wound area. IL-1α and -β as well as TNF-α are strongly up-regulated during the first hours after wounding,[48] possibly interfering with TGF-β signaling early on.

Furthermore, at early stages of wound healing, few keratinocytes and fibroblasts will have direct contact in the early, still poorly populated granulation tissue. Later, the cellular density increases massively and it is conceivable that direct cell-cell contacts or close proximity may occur because of the still regenerating basement membrane zone. This may induce TGF-β synthesis and augment appearance of myofibroblasts. After epithelial wound closure, a provisional basement membrane is regenerated and keratinocytes and fibroblasts are separated again. At this time the epithelium seems to trigger involution of the granulation tissue by still poorly defined mechanisms.[12] Interestingly, in clinical medicine coverage of open wounds with split thickness skin or cultured keratinocyte transplants influences the underlying granulation tissue. In chronic wounds with delayed healing characteristics and poorly developed granulation, there is activation of the underlying dermis with proliferation of dermal cells. In well-healing wounds, transplanted keratinocytes seem to limit and even induce involution of the granulation tissue.

For the early and intermediate stages of normal granulation tissue formation we propose the model outlined in Figure 9. Early, an inflamed fibroblast phenotype shown on the left side of the diagram prevails, which later, as inflammatory activity subsides, differentiates into a myofibroblast. We show for the first time, that keratinocytes induce dermal fibroblasts to differentiate into myofibroblasts. This novel mechanism of keratinocyte-driven myofibroblast differentiation described here requires endogenous TGF-β activity and can be antagonized by keratinocyte-derived or exogenous IL-1. In the physiological context of early stages of wound healing, platelets and inflammatory cells might be the principal inducers of fibroblast activation without myofibroblast differentiation. Later, after the inflammatory phase, epidermal-dermal cell interactions become prominent in driving the wound-healing response. At this time, myofibroblasts appear and become the main ECM producers. This requires a shift from a primarily proinflammatory milieu at the beginning of wound healing to a more TGF-β-dominated one later.

Acknowledgments

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